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14. ABSTRACT

The purpose of our experiments is to identify methods of preventing alloimmunization to donor platelets in a dog platelet transfusion model. We ave established DLA Class II typing to select antigen incompatible donor-recipient pairs for our transfusion experiments. In addition, we are evaluating potential allostimulatory WBC that must be removed to prevent platelet alloimmunization or WBCs that must remain to induce tolerance to donor platelet transfusions. Flow cytometry techniques using antisera that detect various classes of WBCs are being used to identify cells that are removed versus those that remain using different leukoreduction filters. The result of these white cell characterization studies will be correlated with the results of donor platelet transfusion experiments where different filters have been used to leukoreduce donor platelets prior to transfusion.
Our next platelet transfusion experiements will evaluate γ-irradiation alone or combined with leukoreduction as methods of preventing alloimmunization to donor platelets.

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Platelets, platelet transfusion, leukoreduction, γ-Irradiation, alloimmunization, tolerance.

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INTRODUCTION:

The purpose of our research is to identify the contaminating allostimulatory as well as suppressor white blood cells (WBCs) that result in either alloimmunization or tolerance, respectively, to transfused platelets in a dog platelet transfusion model. In addition, we are interested in further exploring the ability of gamma (γ)-irradiation to prevent residual alloimmunization to filter leukoreduced platelets.

KEY ACCOMPLISHMENTS:

- We have confirmed our ability to type dogs for DLA Class II antigens
- We have progressed in our studies to use flow cytometry to identify different types of dog white cells.
- We have identified that the wrong method of isolating dog platelets for transfusion has been done. This problem has been rectified, and we are moving forward with our donor platelet transfusion experiments.

PROGRESS REPORT BODY:

Although funding for this project started on September 1, 2007, we had to hire staff, establish the laboratory procedures, and obtain IACUC approval from the University of Washington Vivarium where our animals are housed before we could proceed with our studies. Our studies also require that we type all the donor and recipient dogs for dog leukocyte antigens (DLA) to ensure that donors and recipients are mismatched for some, if not all, DLA antigens. Typing requires that we obtain samples from available dogs from our animal supplier.

This progress report is divided into three parts:

Progress On Identifying Allostimulatory White Blood Cells – Drs. Karen Nelson and Yvette Latchman:

Comparison of Filters.

Our previous observations had shown a marked increase in the number of recipients who became alloimmunized following transfusion with products filtered with filter 1 compared to filter 2. Our approach has been to study the residual white blood cells (leukocytes) in the platelet products from these two filters using fluorescently-labeled monoclonal antibodies to cell surface proteins of canine leukocyte subsets in order to identify the immunizing cell types. These filter characterization studies were done on blood pooled from three dogs. Ten different pools from the three dogs were then filtered with each of the two filters. Thus, the results achieved with the filter characterization studies were considered statistically very reliable.

Observations this year confirmed earlier results in the types of residual leukocytes in the filtered platelets. However, the novel observation this year was the amount of cell debris with clearly identifiable cell surface antigens present in the platelets filtered with filter 1 that routinely leads to alloimmunization as compared to the other filter that results in a much lower incidence of alloimmunization. The hypothesis we currently are testing is that: shear flow through the filter shatters cells adhering to the filters; the shattered membranes form micelles that persist in the filtered product; recipient dendritic cells ingest this debris; and the recipient is sensitized through indirect presentation of donor DLA.

Progress On DLA Typing – Dr. Lakshmi Gaur:

To date, classical Class I alleles have not been well characterized in dogs. Therefore, Canine major histocompatibility complex Class II (DLA-DRB and DLA-DQB) genes were evaluated. We have developed a PCR based oligotyping method to assign DLA-DQB and DLA-DRB genes utilizing multiple sequence specific polymorphic stretches as oligo-probes that are immobilized on Nylon membranes. The probes were selected from the second exon, as it hosts most of the allele specific polymorphisms in all Class II loci. For each locus, we used exon specific primers for amplification, and the amplification product was hybridized to the nylon membrane with corresponding allele specific probes. Over 40 healthy, previously non transfused and non pregnant dogs were typed in this assay for this project, and the donor-recipient pairs were selected based on MHC mismatching. With the availability of DLA-DRB and DLA-DQB typing of donors and recipients, we have been able to monitor response to DLA "potentially" compatible *versus* incompatible donors."

Progress On Preventing Platelet Alloimmunization – Dr. Sherrill Slichter:

The major hypothesis behind our studies is that γ–irradiation combined with leukofiltration will prevent platelet alloimmunization, even if used with a filter that results in high immunization rates when used as the only method of reducing the immunogenicity of the transfused platelets. In our prior studies, we had only performed three experiments demonstrating the success of the combined γ–irradiation plus filtration process.

γ-Irradiation Experiments.

Previously, we had not yet performed experiments using only γ–irradiation to prevent platelet alloimmunization. Thus, our first experiments were performed to document that γ–irradiation alone did not prevent platelet alloimmunization.

The experimental design of our transfusion experiments is to do 2-3 baseline autologous radiolabeled platelet recovery and survival experiments to document that the recipient animal has normal autologous recoveries and survivals. Next, the treatment under evaluation is then applied to the autologous platelets, the treated platelets are then radiolabeled and transfused. These experiments are done to ensure that the treatment is not injurious to the platelets. Serial post-transfusion blood samples are drawn from the recipient animal after transfusion of either autologous or donor platelets to document the recovery and survival of the radiolabeled platelets. Thus, decreased recoveries and survivals of donor platelets can be attributed to alloimmunization rather than to a recipient animal that is compromised because autologous platelet recoveries and survivals are not normal or that damage has occurred to the donor platelets caused by the treatment. The donor dog's platelets are modified by the treatment under evaluation, radiolabeled, and transfused weekly for up to 8 weeks or until alloimmunization is documented by demonstrating that less than 5% of the donor's platelets remain in the recipient's circulation at 24 hours post-transfusion. After completion of the donor dog transfusions, repeat autologous radiolabeled platelet recovery and survival measurements are performed to document that any refractoriness to the donor dog's platelets is caused by alloimmunization rather than a change in the condition of the recipient animal. In addition, weekly blood samples are drawn from the recipient animal to test for antibody activity against the donor's platelets and lymphocytes compared to the results of the same serum samples tested against autologous platelets and lymphocytes.

Six recipient animals received γ-irradiated platelet transfusions from the same donor animal who was DLA-mismatched with the recipient. The autologous radiolabeled platelet recovery and survival measurements of these 6 recipient dogs are shown in Table 1. These data clearly demonstrated that all 6 recipients had normal radiolabeled autologous platelet recovery and survival measurements both at baseline and post-transfusion of donor platelets. In addition, 4 tested recipients also demonstrated normal autologous platelet recovery and survival measurements after their platelets were treated with γ–irradiation. γ–irradiation experiments were not performed with the last two recipient animals as we considered the data from the first 4 animals sufficient to demonstrate that γ–irradiation did not damage the platelets. As expected, 4 of the 6 recipients became immunized to the donor's γ–irradiated platelets after a single transfusion. The results on the other 2 recipients were not interpretable due to inadequate labeling of the donor platelets with the radiochromium used to label the donor platelets. Radiochromium was not available from our usual supplier and so an alternate source was used which did not give reliable labels.

γ-Irradiation Plus Leukofiltration Experiments.

Two recipient dogs have been entered into γ–irradiation, leukofiltration experiments. Their autologous radiolabeled platelet recovery and survival data are shown in Table 2. Again, the baseline radiolabeled autologous and post-treatment autologous experiments gave normal platelet recovery and survival data. However, unexpectedly, 1 of the 2 recipients became alloimmunized to the donors' γ–irradiated, filter leukoreduced platelets. In reviewing the potential causes for this, we discovered that the methods we had been using to prepare the donors' blood for transfusion were based on methods used for our human radiolabeling studies that use a tube method of separating platelets from whole blood rather than the bag method we had always previously used for our dog experiments. The tube separation method, when used in the dog, gave a much higher residual WBC contamination level than with the bag method. This means that the filter may not have been adequate to remove the required number of WBCs. Indeed, in reviewing the data, the residual WBC level was 5 to 10 times higher when the tube method of platelet preparation was used compared

to when the bag method was used. Unfortunately, when our prior dog radiolabeling technician left and we hired a replacement technician to perform these studies, the initial technician gave the replacement technician the wrong platelet preparation method. Therefore, all of the studies performed to date will have to be repeated.

REPORTABLE OUTCOMES:

A manuscript on the DLA typing methods we have developed in the dog is in preparation.

CONCLUSION:

None.

REFERENCES:

None.

APPENDICES:

None.

SUPPORTING DATA:

TABLE 1

RECIPIENTS RECEIVING γ-IRRADIATED DONOR PLATELET TRANSFUSIONS

Recipient Number	AUTO BASELINE			AUTO γ-IRRADIATED			AUTO POST-DONOR TRANSFUSIONS		
	N	Platelet Recovery (%)	Platelet Survival (Days)	N	Platelet Recovery (%)	Platelet Survival (Days)	N	Platelet Recovery (%)	Platelet Survival (Days)
4919	3	51 ± 22	5.2 ± 0.8	3	45 ± 6	4.7 ± 2.6	3	50 ± 7	6.9 ± 2.6
6061	3	67 ± 6	6.6 ± 0.7	2	61 ± 5	5.3 ± 1.3	2	63	5.4 ± 0.7
6275	3	56 ± 11	5.6 ± 2.8	2	59 ± 0	5.5 ± 1.9	3	64 ± 8	4.9 ± 0.2
5250	3	55 ± 3	5.8 ± 1.9	2	51 ± 1.4	5.5 ± 1.9	2	51 ± 2.8	5.3 ± 1.5
863	2	67 ± 3	4.0 ± 0.2	---	ND	ND	3	69 ± 18	4.2 ± 1.7
6041	2	68 ± 9	4.4 ± 0.8	---	ND	ND	2	65 ± 3	4.3 ± 0.2

Data reported as average ±1 S.D.

TABLE 2

RECIPIENTS RECEIVING γ-IRRADIATED AND FILTER LEUKOREDUCED DONOR PLATELET TRANSFUSIONS

Recipient Number	AUTO BASELINE			AUTO POST-DONOR PLATELET TRANSFUSIONS		
	N	Platelet Recovery (%)	Platelet Survival (Days)	N	Platelet Recovery (%)	Platelet Survival (Days)
0335	2	49 ± 11	5.2 ± 0.8	2	65 ± 4	4.0 ± 0.4
276	2	46 ± 0	4.1 ± 0.4	1	56	3.8

Data reported as average ±1 S.D.